Etomidate reduces initiation of backpropagating dendritic action potentials: implications for sensory processing and synaptic plasticity during anesthesia

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Running head: Reduced dendritic backpropagation under etomidate

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Abstract

Anesthetics may induce specific changes that alter the balance of activity within neural networks. Here we describe the effects of the GABA$_A$-receptor potentiating anesthetic etomidate on sensory processing, studied in a cerebellum-like structure, the electro-sensory lateral line lobe (ELL) of mormyrid fish, in vitro. Previous studies have shown that the ELL integrates sensory input and removes predictable features by comparing reafferent sensory signals with a descending electromotor command-driven corollary signal that arrives in part via parallel fiber synapses with the apical dendrites of GABAergic interneurons. These synapses show spike timing dependent depression when presynaptic activation is associated with postsynaptic backpropagating dendritic action potentials.

Under etomidate almost all neurons become tonically hyperpolarized. The threshold for action potential initiation increased for both synaptic activation and direct intracellular depolarization. Synaptically-evoked IPSPs were also strongly potentiated and prolonged. Current source density analysis showed that backpropagation of action potentials through the apical dendritic arborization in the molecular layer was reduced, but could be restored by increasing stimulus strength. These effects of etomidate were blocked by bicuculline or picrotoxin. It is concluded that etomidate affects both tonic and phasic inhibitory conductances at GABA$_A$ receptors and that increased shunting inhibition at the level of the proximal dendrites also contributes to increasing the threshold for action potential backpropagation. When stimulus strength is sufficient to evoke backpropagation, repetitive association of synaptic excitation with postsynaptic action potential initiation still results in synaptic depression, showing that etomidate does not interfere with the molecular mechanism underlying plastic modulation.
Introduction

The anesthetic and sedative actions of many general anesthetics are related to their ability to potentiate the activity of GABA at GABA_A-receptors. While behavioral and c-FOS expression studies have localized the sedative actions of GABAergic anesthesia to GABA_A receptors in a hypothalamic region involved in sleep control (Nelson et al. 2002), changes of the balance of activity within other neural networks have also been observed in, for instance, the hippocampus and cerebellum (Antkowiak and Heck 1997; Faulkner et al. 1998; Dickinson et al. 2003. However, most of the studies on the effects of anesthetics have been devoted to their interactions with GABA_A receptors at the molecular level, and their actions on network and cellular activity remain only poorly understood. In order to obtain a better understanding of anesthetic action on integrative function, we have explored how the anesthetic etomidate influences sensory processing and synaptic plasticity in a cerebellum-like network.

Etomidate is an imidazole, nonbarbiturate hypnotic agent that is increasingly used in procedural sedation (Rothermel 2003) and which, at anesthetic doses, potentiates the activity of GABA at GABA_A receptors (Yang and Uchida 1996) with selectivity for β2 and β3 subunit-containing receptors (Reynolds et al. 2003). Separate sedative and hypnotic effects of etomidate have been distinguished. The sedative effect of etomidate appears to act via the β2 subunit of the GABA_A receptor (Reynolds et al. 2003) and is mediated principally by enhancement of tonic conductance. This has been described in thalamocortical neurons of the ventrobasalis complex that have been implicated in the generation of sleep (Belelli et al. 2005), but the effect of etomidate on different neurons is varied, depending on the subunit composition of synaptic and extrasynaptic receptors. Belelli et al. (2005) also describe potentiation of mIPSPs in both
ventrobasalis neurons and neurons of the thalamic nucleus reticularis, although the latter neurons are not tonically hyperpolarized by etomidate.

Another of the few available reports on the effects of etomidate on neural networks shows that in cortical networks etomidate either depresses or enhances theta wave oscillations, again depending on the type of GABA receptor β-subunit that is present (Drexler et al. 2005). It has also been shown that etomidate reduces cortical cell responsiveness to incoming sensory transmission in the dorsal column nucleus in rats (Angel and Arnott 1999), although the animals in this study also received baseline anesthesia with urethane.

The present experiments have been designed to examine the effects of etomidate on sensory processing and integration with descending modulation signals, and on synaptic plasticity involving GABAergic microcircuits, using as an experimental model the electrosensory lobe of the mormyrid electric fish. The electrosensory lateral line lobe (ELL) contains a cerebellum-like network in which primary afferent input is integrated with descending corollary discharge signals that provide an active filtering mechanism, enabling the network to remove predictable features from reafferent sensory input. The intrinsic circuitry of the ELL is well known (Grant et al. 1996; Meek et al. 1996; Meek et al. 1999; Bell et al. 2005), and closely resembles that of the dorsal cochlear nucleus, with a number of features also common to mammalian cortical structures. Much of the integrative physiology of the electrosensory system has been described in vivo and in vitro, and it has been shown that spike timing dependent plasticity of synaptic responses to parallel fiber input serves to form and update on-going central predictions of electrosensory input (Bell et al. 1997; Han et al. 2000b). Plasticity results from repetitive association of pre- and postsynaptic events and is most clearly expressed in GABAergic interneurons known as Medium Ganglionic layer neurons (MG), in which it depends
on the dendritic backpropagation of characteristically broad action potentials through the apical
dendritic tree in the molecular layer (Bell et al. 1997; Grant et al. 1998; Han et al. 2000b; Gómez
et al. 2005). The present study of the network mechanisms and cellular phenomena underlying
etomidate anesthesia complements a recent exploration of the effects of etomidate anesthesia in
vivo on responses to electrosensory stimuli and modulation of the structure of receptive fields
(Engelmann et al. 2006).

Materials and Methods

A total of 24 Gnathonemus petersii, ranging in length from 8-11 cm, were used for the in
vitro experiments. All research procedures concerning the experimental animals and their care
adhered to the American Physiological Society’s Guiding Principles in the Care and Use of
123.

The procedures to isolate the brain and to prepare 400 µm thick slices have been described
previously in Grant et al. (1998). Briefly, the ELL was collected in ice-cold low-sodium artificial
CSF, containing (in mM): NaCl 0, KCl 2.5, NaH₂PO₄·H₂O 1.25, NaHCO₃ 24, CaCl₂ 2,
MgSO₄·7H₂O 2, glucose 10, and sucrose 210. The tissue was sectioned transversally under ice-
cold low-sodium aCSF with a sapphire knife (DDK, Wilmington, USA) in a Leica 2000
vibratome (Leica, Wetzlar, Germany); the cutting plane was tilted 10° from horizontal. The
slices were collected in low-sodium aCSF at room temperature (23-25°C), and then transferred to
an interface recording chamber. Here they were superfused with a medium-sodium/high-
magnesium solution containing (in mM): NaCl 58, KCl 2.5, NaH₂PO₄·H₂O 1.25, NaHCO₃ 24,
CaCl₂ 2, MgSO₄.7H₂O 2, glucose 10, and sucrose 105. After a further 20 min the superfusion solution was changed to one in which the sucrose was replaced by 116 mM NaCl, for 45 min. Finally, the slices were superfused with normal aCSF composed of (in mM): NaCl 116, KCl 2.5, NaH₂PO₄.H₂O 1.25, NaHCO₃ 24, CaCl₂ 2, MgSO₄.7H₂O 1.2, and glucose 10. All solutions were saturated with a 95% O₂/5% CO₂ gas mixture. The pH was 7.3-7.4 and osmolarity was ~280 mOsm. Perfusion was 1-2 ml/min by gravity flow.

Stimulation

The electrosensory lobe (ELL) molecular layer or the deep fiber layer were stimulated using paired gold-plated tungsten electrodes (Frederic Haer & Co, Bowdoinham, USA) (Fig. 1). Constant current stimulation strength was 3-20 µA and the stimulus cycle repetition interval was 4 seconds.

Recordings

Field potential recordings were made with glass microelectrodes containing 3M NaCl, with resistances of 7-10 MΩ. For Current Source Density analysis (CSD), field potential recordings were made serially at points separated by 25 µm steps through the ELL, starting at the dorsal margin of the molecular layer, through the layers to the deep fiber layer (see dotted line in Fig 1). Current sinks (produced by current flowing into the cells) and sources (produced by current flowing out of the cells) were calculated from averages of 15 field potential traces recorded at each site, to assess current flow through the layers of the ELL. Since the ELL is a laminated structure and is activated homogeneously with respect to the laminar planes (stimulation of primary afferents or parallel fibers induces current flow through the layers in one plane only), the
one-dimensional current source density method can be applied (Mitzdorf 1985, Gómez et al. 2005). CSD is calculated as the second spatial derivative, and is experimentally approximated by:

\[
\frac{\delta^2 V(t)}{\delta x^2} \approx \frac{(V_d - 2V_0 + V_v)}{\Delta x^2}
\]

where \(V_0\) is the field potential for which current source density is calculated at time (t), \(V_d\) is the field potential at time (t) for the point 25 µm more dorsal, and \(V_v\) is the field potential at time (t) for the point 25 µm more ventral to \(V_0\). \(\Delta x\) is the distance between two field potential recording sites: 25 µm in the present experiments. As we did not measure extracellular conductivity, the present CSD analyses give a qualitative rather than a quantitative estimation of current flow. In order to highlight and compare events of low amplitude, the CSD data were multiplied by an amplification factor of 3000, and subsequently transformed by applying an arctangent function (f). This is a sigmoid function where \(f_0 = 0\) and limits are \(f_{\infty - \infty} = -\pi/2\), \(f_{\infty + \infty} = \pi/2\). The result of this transformation is that the resolution of low amplitude events is enhanced. High amplitude events, such as those generated by stimulus artifacts, are not affected, as they were already saturated and cannot increase further (Gómez et al. 2004). In CSD plots, the color code conversion of current values is made with a modified version of the standard \textit{contourf} function of MATLAB (Mathworks, Natick, USA).

Intracellular recordings were made with sharp microelectrodes filled with 2M potassium methyl sulphate (Sigma, Steinheim, Germany) containing 2% biocytin (Sigma, Steinheim, Germany) (resistance 140-240 MΩ) to label the cells that were recorded. Neuron morphology was matched with electrophysiological characteristics of the cell types: efferent cells fire large
narrow spikes while MG cells fire both large broad spikes and small narrow spikes (Bell et al. 1997; Grant et al. 1998).

Etomidate (Janssen-Cilag, Issy les Moulineaux, France) was bath-applied at 4.1 µM, which is the concentration that has been used to produce deep surgical anesthesia of the fish for in vivo experiments (Gómez et al. 2004). The action of GABA at GABA<sub>A</sub>-receptors was blocked using bicuculline (30 µM) or picrotoxin (20 µM) (Sigma, Steinheim, Germany). D-2-amino-5-phosphonopentanoate (AP-5, Sigma, Steinheim, Germany) was bath-applied at 40 µM to block NMDA-receptor activation in some experiments.

To analyze whether etomidate modulates spike-timing dependent plasticity, the protocol described by Han et al. (2000b) was used to induce depression at parallel fiber synapses with the apical dendrites of ganglionic layer neurons. Briefly, a synaptically-evoked, subthreshold EPSP was paired with backpropagating action potentials evoked by an intracellular stimulus delivered after a delay of 25 ms. The stimulation frequency was 0.2 Hz, and pairing lasted for 6 min.

Data acquisition and analysis

Data were stored after amplification (Axoclamp 2B amplifier, Axon Instruments, Foster City, USA) using Elphy acquisition software (G. Sadoc, CNRS, France) and analyzed off-line. CSD plots were made using Matlab software (Mathworks, Natick, USA). Statistical comparisons were made with the paired Student’s t test, unless indicated otherwise.

Results
The effect of etomidate on sensory input (deep fiber layer stimulation)

Stimulation in the deep fiber layer of the ELL evokes a single large negative field potential recorded in the layer itself, that most likely corresponds to the activation of primary afferent fibers (“n pre” in Fig. 2e) (Grant et al. 1998), being very similar to the primary afferent volley recorded in vivo in response to the fish’s electric organ discharge (Engelmann et al. 2006). Primary afferent fibers terminate in a restricted, topographically organized manner in the overlying granular layer and here, the response to the same stimulation is a two-peaked negative field potential that probably reflects the presynaptic activity of the primary afferents (n pre) followed by the postsynaptic response of their target granular cells (n post) (Fig. 2d).

When the deep fiber layer stimulation is four times the threshold for responses in the granular cell layer, a prominent double negative wave response is also evoked in the overlying plexiform and ganglionic layers (Fig. 2c). These layers contain the terminal arborisation of superficial granular layer neurons, the basal dendrites and cell bodies of efferent large fusiform (LF) and large ganglionic layer (LG) cells, and also the soma of interneurons known as medium ganglionic layer cells (MG) which constitute up to 80% of the total cell population in the ganglionic cell layer. LG, LF and MG cells all extend apical dendrites in a regular array into the overlying molecular layer, as illustrated in Fig. 1.

The negative waves originating in the plexiform and ganglionic layers, termed “early” (ne) and “late” (nl), propagate outwards through the molecular layer (Fig. 2a,b). It was suggested previously that nl corresponds to the synchronous backpropagation of action potentials through the apical dendritic tree of MG cells, which have characteristically broad action potentials lasting up to 10 ms (Grant et al. 1998; Gómez et al. 2005). The amplitude of both nl and of the corresponding backpropagating current sink (Fig. 3, see below) diminishes with distance,
although it extends to the outer limit of the molecular layer. This corresponds to the anatomy of ganglionic layer neurons, and in particular MG neurons, whose apical dendrites extend across the whole depth of the molecular layer (Fig. 1).

Current source density analysis (Fig. 3A) shows that n pre and n post are associated with strong current sinks in the deep fiber and granular layers. nl is associated with a current sink originating in the ganglionic layer, that then propagates out through the molecular layer. These waves are accompanied by a complex current source extending through the lower ganglionic cell layer, the plexiform and the superficial granular layers. The early part of this current source appears to be coupled with the sink in the deep granular, intermediate and deep fiber layers, whereas the later part is linked to the backpropagating sink associated with nl in the molecular layer.

When successive stimuli are delivered to the deep fiber layer with an interval of 50 ms, backpropagation of activity in the molecular layer is markedly depressed in response to the second stimulus (see Fig 3 A,F). The example illustrated in Fig. 3F shows that after the second stimulus the amplitude of the nl field potential (measured at the level indicated by the dotted white line in Fig. 3A) was reduced to 70.1 ± 12.7% of the response to the first stimulus (p < 0.05; n = 4). The duration of the backpropagating sink (again measured at the level of the arrow in Fig. 3A) was also reduced from 5.6 ± 0.8 ms after the first stimulus to 4.1 ± 0.3 ms after the second stimulus (p < 0.05; n = 4). As a consequence of the paired-pulse depression seen in the field potentials, the sink corresponding to the propagation (nl) in the CSD is strongly attenuated (Fig. 3A) and the later part of the current source in the ganglionic cell layer was essentially absent following the second stimulus, although the sink in the granular and lower layers remained unchanged (p = 0.89; n = 4, Fig. 3A). This confirms that this later ganglionic/plexiform layer
current source is coupled to the backpropagating molecular layer sink and not to the sink in the deep fiber and granular cell layers.

Etomidate applied in the bath solution, at a concentration that produces deep anesthesia in vivo (4.1 µM), markedly reduced backpropagating activity in the molecular layer (Fig. 3B). The nl-wave amplitude was depressed by 88 ± 6% after the first stimulus (Fig. 3F; p < 0.05; n = 4) and only a very small response was obtained to the second stimulus. The corresponding late source in the plexiform and upper granular layer also disappeared. However, the early source and sink associated with primary afferent activity in the deeper layers and the synaptic response of granular cells were not affected. Paired-pulse depression persisted in the presence of etomidate, especially evident for the backpropagating sink in the molecular layer, demonstrating that paired-pulse depression does not depend on GABA_A receptor activity.

The GABA_A receptor antagonists bicuculline or picrotoxin, applied in addition to etomidate, restored backpropagation, suggesting strongly that etomidate had blocked backpropagating events through potentiation of inhibition acting at GABA_A receptors on the soma or apical dendrites of ganglionic layer neurons (Fig. 3C).

Interestingly, in the deep fiber layer, bicuculline increased the duration of the primary afferent-related current sink (white arrow) from 3.4 ± 0.3 ms to 10.2 ± 0.7 ms (p < 0.05; n = 4), and following low amplitude activity which was still visible up to 30 ms after the stimulus, suggesting that postsynaptic responses to the primary afferent volley might be prolonged. This indicates that the initial excitatory response to deep layer stimulation is normally followed rapidly by strong GABAergic inhibition. Since this primary afferent-related current sink was not altered under etomidate alone (Fig. 3B) it can be inferred that GABAergic inhibition is normally maximal in the deep fiber layer under control conditions. Picrotoxin similarly increased the
duration of this sink, confirming that the effect evoked by bicuculline can be attributed to its action on GABA_A receptors rather than to interaction with calcium- and voltage-gated potassium channels (data not shown; n = 2). However, it is also possible that the GABA_A receptor subunit composition of primary afferents or granular cells excludes any inhibitory effects of etomidate.

Paired-pulse depression persisted in the presence of bicuculline or picrotoxin, in both the deep fiber layer and the molecular layer. Since under these conditions GABA_A receptors are blocked, it must be concluded that paired pulse depression is not the direct result of inhibition acting through GABA_A receptors, confirming the results of Han et al. (2000a). Washout of first bicuculline (Fig. 3D) and then etomidate (Fig. 3E) shows that the effects induced by etomidate and bicuculline are reversible, although we observed that it often took several hours to obtain good etomidate washout in this isolated preparation, in contrast with the rather rapid recovery observed in vivo (Engelmann et al. 2006).

While these results are most readily observed in current source density plots which show an ensemble view of network activity, the same effects of etomidate, and etomidate with bicuculline, can be seen in the field potentials shown in Fig. 3F, recorded in the molecular layer at the level indicated by a white dotted line in Fig. 3A.

The reduced amplitude of the backpropagating sink could reflect either reduced initiation of backpropagating spikes, or failure of broad spikes to backpropagate in MG cells. To test this, a weak deep fiber layer stimulus was applied initially, sufficient to produce a backpropagating sink (Fig. 4A) but below the level of response saturation. The backpropagating sink was then reduced under etomidate (Fig. 4B) but could be restored by doubling the stimulus intensity (Fig. 4C; n = 3). This shows that backpropagation was not blocked _per se_ but that the threshold for initiation of backpropagating events was increased under etomidate.
The effect of etomidate on descending input (molecular layer stimulation)

Stimulation in the ELL molecular layer (Figs. 5 and 6) activates parallel fibers running through the molecular layer which synapse with the apical dendritic arborisation of ganglionic layer neurons and with GABAergic stellate cells intrinsic to the molecular layer (Fig.1). This stimulation evokes three negative field potentials in the molecular layer (Fig. 5a), accompanied by a positive wave in the ganglionic and plexiform layers containing the somata and basal dendrites of the LG, MG and LF cells (Fig. 5b,c). It has been suggested by previous authors (Grant et al. 1998, Gómez et al., 2005) that these waves represent the parallel fiber volley (n1), activation of synapses on the apical dendrites of ganglionic layer neurons (n2), and synchronously generated backpropagating broad spikes fired by MG cells (n3). Paired pulse stimulation produced marked facilitation of these field potentials, in particular n3. The amplitude of n2 increased to 132 ± 15% (p < 0.01; n = 8), and n3 became clearly visible.

CSD analysis (Fig. 6) shows that the n2 field potential is associated with a strong current sink in the molecular layer, occurring at the level of, and distal to, the incoming parallel fiber beam, together with a corresponding current source extending through the inner molecular layer and deeper layers (Fig. 6A). This is followed by a current sink in the ganglionic layer, which then appears to propagate out through the molecular layer, associated with the n3 negative wave of the field potential recorded “on beam” with the parallel fiber input. The backpropagating sink corresponding to n3 reflects the synchronous backpropagation of action potentials through the apical dendritic tree of ganglionic layer neurons, of which MG cells form a large majority. The apparent amplification of the current sink at the level of the parallel fiber input (Fig 6A and C,
white arrow) suggests that coincidence of backpropagating action potentials with the NMDA component of the synaptic response might cause a local active event in the distal dendrites.

Etomidate essentially abolished backpropagation of dendritic events from the ganglionic layer through the molecular layer, and thus also the n3 field potential, although the initial genesis of a late current sink in the ganglionic cell layer was maintained and continued to show paired pulse facilitation (Fig. 6B). The earlier strong sink corresponding to synaptic activation (n2) in the molecular layer became a little longer, possibly because it was no longer masked by the source/sink/source constellation characteristic of the backpropagating action potentials (see Mitzdorf 1985).

Bicuculline restored backpropagation of dendritic events from the ganglionic layer through the molecular layer, and indeed amplified synaptically induced current sinks in both the ganglionic and molecular layer beyond control levels (Fig. 6C; n=4). Bath-application of picrotoxin yielded similar results (n=2; not shown). This confirms that the action of etomidate was mediated through GABA\textsubscript{A} receptors, and also underlines the mixed excitatory and inhibitory nature of synaptic responses to parallel fiber stimulation under normal conditions.

Apical dendritic trees in the molecular layer receive many GABAergic synapses (Meek et al. 1996). Thus the effect of etomidate in reducing backpropagation in response to molecular layer stimulation might be produced by both an increase in the threshold for action potential initiation and increased shunting inhibition of synaptic origin at the level of the proximal dendrites. This possibility was addressed by applying a weak stimulus, sufficiently strong to elicit backpropagation (Fig. 7A) but without saturation of the response. Etomidate reduced the backpropagating sink (Fig. 7B), but this could be at least partially restored by doubling the stimulus strength (Fig. 7C, response to second stimulus; n = 3). This suggests that the threshold
for backpropagation was increased under etomidate but shows that etomidate did not completely block the mechanism for backpropagation.

However, in figure 7C, even though the parallel fiber stimulus was twice as strong as in the control (Fig. 7A), the backpropagating sink is only slightly more evident than in figure 7B. There are several possible reasons for this.

When the parallel fiber stimulus was stronger and evoked a larger postsynaptic response in the distal dendrites, the flanking sources were also stronger. The intensity of the source separating the sinks in the inner and outer molecular layer may then be sufficient to mask any backpropagating sink. An alternative explanation is that potentiation of inhibitory synaptic input to the apical dendritic tree increases shunting inhibition at the level of the proximal dendrites and thus action potentials fail to backpropagate. Shunting inhibition of this type has been described in cerebellar Purkinje cells, where inhibitory input from stellate cells reduces the amplitude of calcium spikes (Callaway et al., 1995). If this is the case, then the increased amplitude of the molecular layer sink “on beam” with the parallel fiber input, must be simply the consequence of prolonged distal synaptic activity, especially of the NMDA receptor component. The duration of the NMDA component of the postsynaptic response was illustrated by bath-application of AP-5 (40 µM), which strongly reduced the late phase of the current sink in the outer molecular layer (Fig. 8 white arrow).

To explore the mechanisms underlying the effects of etomidate on backpropagation in further detail, we studied the effects of this anesthetic in individual cells.
Cellular responses to parallel fiber stimulation

Resting membrane potential was similar (p = 0.94) for MG cells and for efferent neurons, in the range of 64.5 ± 3.4 mV (means ± se; n=19). All MG and efferent cells (n = 19) showed an EPSP following parallel fiber stimulation, and this was followed by a visible IPSP in 12 cells (Fig. 9).

Etomidate tonically hyperpolarized 18 of the 19 cells tested, by 2 – 4 mV (Fig. 9A and C), giving a mean membrane potential of -67.9 ± 2.7 mV (mean ± se). Only a single LF cell was not tonically hyperpolarized. In parallel, input resistance decreased by 11.6 ± 0.8% (from 35.0 to 30.9 MΩ on average), tested in one MG cell and three LG cells. In the single LF cell recorded, input resistance decreased only very slightly (2%), consistent with the absence of tonic hyperpolarization. Thus, it appears that GABA is constantly present in the slice preparation, and that etomidate potentiated tonic GABAergic activity in all LG and MG cells tested.

Etomidate markedly potentiated IPSPs in all cells in which they were visible initially and also unmasked inhibitory responses in cells where no IPSP was initially apparent. This demonstrates that etomidate also potentiates phasic GABA receptors, in both interneurons and projection neurons. In five MG and seven LG cells in which an EPSP - IPSP sequence was already apparent, etomidate prolonged the IPSP up to 300 ms (Fig. 9C). An IPSP developed under etomidate in four MG, one LF, and two LG cells in which only an EPSP was visible in control conditions (Fig. 9A and B). In some cases the earlier EPSP appeared to be shunted by the increased following IPSP, as illustrated for the LF and LG cells in Fig. 9B and C. These results show that efferent cells and inhibitory interneurons with apical dendrites projecting through the molecular layer all receive both excitatory and inhibitory input following parallel fiber
stimulation, and that the resulting postsynaptic potential is a composite event, in which the inhibitory component is amplified under etomidate.

Bicuculline antagonized the potentiated inhibition induced by etomidate, and effectively blocked IPSPs generated by parallel fiber stimulation (Fig. 9D). This confirms that the inhibitory input is mediated via GABA\textsubscript{A} receptors. The threshold for synaptically evoked postsynaptic action potential generation was also reduced under bicuculline, suggesting that even in control conditions spiking threshold is controlled by tonic inhibition. Compared with the normal condition, the pharmacological blockade of GABA\textsubscript{A} receptors removed shunting inhibition and allowed the membrane to reach the threshold for spiking faster, resulting in earlier action potential firing (a shift of 0.6 ± 0.07 ms). Action potentials were also larger under bicuculline, no longer being shunted by the following disynaptic IPSP (amplitude of the first spike increased from 61 ± 2 mV to 67 ± 3 mV), and the cell was able to fire more than one action potential in response to molecular layer stimulation (Fig. 9D; tested in two MG and four LG cells).

Intracellular records showed that reduced neuron excitability under etomidate was due to both increased tonic hyperpolarization and to potentiation of synaptic inhibition and that increased shunting inhibition at the level of the proximal dendrites very probably played a role in preventing the backpropagation of dendritic action potentials.

This was explored in four MG and three LG cells, looking at responses to both intracellular depolarization and parallel fiber synaptic input. Figure 10A1 shows an MG cell in which parallel fiber stimulation evoked an EPSP, giving rise to a spikelet followed by a broad action potential and then a following IPSP. Intracellular stimulation evoked a spikelet followed by a broad action potential. Note that spikelets have been identified as axonal spikes in a previous publication (Grant et al. 1998).
Under etomidate (Fig. 10A2 and A3) the membrane potential became tonically hyperpolarized, the broad spike was reduced in amplitude and then failed as it was shunted by the potentiated disynaptic IPSP, and the IPSP was markedly prolonged. The response to an intracellular depolarization given at a delay of 80 ms also failed, probably due to both potentiated phasic conductance and increased shunting inhibition at the level of the proximal dendrites. Doubling stimulus strengths restored both the synaptic and intracellularly evoked action potentials (Fig. 10B1 and B2), showing that while the threshold was increased, the basic mechanism for action potential initiation was not blocked. The importance of the potentiated synaptic inhibition in preventing the initiation of a backpropagating broad spike is confirmed by the counter-example in figure 10B3, where an intracellular depolarization of the same strength as the initial control was given without a preceding parallel fiber stimulus and in this case evoked a backpropagating action potential. Tonic hyperpolarization was then balanced out by repolarizing the membrane to the initial resting potential (Fig. B4) and this restored the spiking response to the parallel fiber stimulus, although spike amplitude was reduced due to shunting inhibition. However the same intracellular depolarization given at 80 ms was not able to produce an action potential, revealing the importance of potentiated synaptic inhibition in preventing backpropagation.

The impact of tonic hyperpolarization and of the potentiation of synaptic IPSPs on action potential firing was also noted in LG efferent neurons (Fig. 10C). In the control situation, an intracellular depolarization following synaptic activation at a delay of 20 ms generated a burst of 4 action potentials (Fig. 10C1). Under etomidate, only a single action potential was generated by the same stimulus (Fig. 10C2). However, when the delay between the parallel fiber stimulus and the intracellular depolarization was increased to 55 ms, the neuron again fired a burst of 4 action
potentials (Fig. 10C3). This demonstrates that the tonic hyperpolarisation induced by etomidate was not sufficient, alone, to completely block action potential initiation in all 3 cells tested, and illustrates the significance of the increased duration of the parallel fiber IPSP under etomidate.

**Plasticity**

Associative, spike timing dependent, inverse Hebbian plasticity at the parallel fiber to MG cell synapse has been documented in detail in previous studies (Bell et al. 1997; Han et al. 2000b). Briefly, expression of spike timing dependent synaptic depression requires that a postsynaptic backpropagating action potential occur within a window of about 60 ms following activation of the parallel fiber input. The plastic change in synaptic efficacy involves the NMDA component of the parallel fiber postsynaptic response and also depends on postsynaptic intracellular calcium. Functionally, this is a mechanism by which an internal expectation driven by the corollary discharge pathway is updated in the case of a predictable sensory input (Bell, 2001). A previous study has demonstrated reduced expression of plasticity of corollary discharge input to MG neurons in vivo, in animals anaesthetized with etomidate (Engelmann et al. 2006). Since the present in vitro study has shown that etomidate reduces the initiation of backpropagating action potentials, it was particularly interesting to test for plasticity, in order to better understand its action related to anesthetic properties.

Spike timing dependent depression of excitatory input was induced in MG cells by pairing a subthreshold parallel fiber EPSP with a postsynaptic broad spike that was evoked by an intracellular depolarizing pulse 25 ms following EPSP onset, using the protocol published in several previous studies (Bell et al. 1997; Han et al. 2000b). As a control to demonstrate that plastic change involved principally the excitatory component of the synaptic response, the
associative pairing protocol was first applied under etomidate with bicuculline to block the disynaptic inhibitory component of the response (Fig. 11A). Following pairing, EPSP amplitude was reduced (n= 2), giving the same result as in previous studies (Bell et al. 1997; Han et al. 2000b). However, since etomidate acts on GABA_A receptors, it is necessary to carry out the experiment in the absence of bicuculline, using a protocol similar to that published in Bell et al. (1997). In the absence of bicuculline, the postsynaptic response to the parallel fiber stimulus is a mixed excitatory/inhibitory sequence. Figure11B (black trace) shows a control response to parallel fiber stimulation under etomidate, before any pairing protocols. Following pairing, the excitatory component of the response was reduced by 26 ± 6 % (mean ± se; n = 5 cells from 5 fish; p < 0.05; Kolmogorov-Smirnov; gray trace). This unmasked the early onset of the disynaptic IPSP. In the absence of further pre- and postsynaptic association, the response to parallel fiber input gradually returned towards control values over about 10 minutes (Fig. 11C), as has been described in the previous studies cited above. Thus, spike timing dependent plasticity at the parallel fiber synapse with MG neurons can be demonstrated in the presence of etomidate, as in the control situation (see Bell et al. 1997).

Discussion

Current source density analysis showed that the major effect of etomidate in the ELL was to reduce backpropagation of activity through the apical dendritic arborisation of ganglionic layer neurons. This was due to an increase in the threshold for firing of backpropagating action potentials, caused by both membrane hyperpolarisation and potentiation of synaptic inhibition. This effect of etomidate has implications for plastic modulation of sensory integration since
earlier studies have shown that the expression of plasticity at parallel fiber synapses depends on
the generation of a backpropagating action potential occurring within a window of up to 60 ms
following presynaptic activation (Bell et al. 1997; Han et al. 2000b). Our recent in vivo studies
demonstrated that etomidate anesthesia indeed affects plasticity, but this effect may have been
influenced by the slower electric organ discharge rate under etomidate, which reduces the
number and frequency of pairings during a given period (Engelmann et al. 2006).

The present results indicate that etomidate indeed affects plasticity, acting directly within
the ELL by inhibiting firing of backpropagating spikes, rather than by blocking the underlying
molecular mechanisms of plastic change at parallel fiber-to-MG cell synapses. Intracellular
records have shown that association of parallel fiber input with backpropagating action
potentials, generated by imposed depolarization, can still produce spike timing dependent
synaptic depression under etomidate. The exact mechanism involved in spike timing dependent
plasticity has not yet been completely identified in the ELL, but is known to depend on NMDA
receptor activation and increased postsynaptic calcium (Han et al. 2000b). The present CSD
analysis has demonstrated local amplification of distal dendritic events, generated by coincidence
of the backpropagating action potentials with the NMDA component of the dendritic
postsynaptic response. This suggests that a strong local depolarization at this level, sufficient to
unblock voltage dependent block of NMDA receptor channels by Mg\(^{2+}\) (Kampa et al. 2004), may
be the necessary trigger for plastic change.

The effects of etomidate were blocked by either bicuculline or picrotoxin, confirming that
its action is mediated through GABA\(_A\) receptor channels. Previous studies have shown that
etomidate binds to GABA\(_A\) receptors, increasing the probability of Cl\(^-\) channel opening and
prolonging their open state (Yang and Uchida, 1996). At anesthetic concentrations, etomidate
potentiates the inhibitory activity of GABA, but does not exert any direct effect by itself. At higher concentrations, which have been reported in the range of less than 10 µM (Hong and Wang 2005) up to 80 µM (Yang and Uchida 1996), etomidate has GABA-mimetic effects, and activates chloride currents directly. The concentration that was used in the present experiments (4.1 µM), corresponding to the dose that produces deep surgical anesthesia in vivo (Gómez et al. 2004), is very probably below the threshold for GABA-mimetic effects, considering that the chloride current induced by application of 10 µM of etomidate is very small (Hong and Wang 2005). Therefore it is our interpretation that most, if not all, of the effects observed in the present study were due to potentiation of the inhibitory action of GABA.

In the present experiments, intracellular records showed that under etomidate almost all ELL neurons became tonically hyperpolarized and that input resistance decreased, suggesting an action on tonic GABA<sub>A</sub> receptors (Farrant and Nusser 2005), increasing chloride ion permeability and moving the membrane potential closer to the chloride equilibrium potential. By removing the membrane potential further from the action potential firing threshold, this affected the probability of firing, especially of high-threshold backpropagating dendritic spikes, in response to excitatory synaptic input.

In the ELL, excitatory input, whether of sensory or central origin, is almost always followed by short latency inhibition and the present results show that in addition to a tonic hyperpolarizing action, etomidate also markedly increased the amplitude and duration of synaptic inhibitory potentials, indicating a parallel action on postsynaptic phasic GABA<sub>A</sub> receptors. Since the somata of ELL efferent neurons in the ganglionic and plexiform layers and the spiny apical dendrites of LG, MG and LF neurons in the molecular layer are densely covered with GABAergic terminals (Grant et al. 1996; Meek et al. 1996), potentiation of IPSPs by
etomidate would have the effect of clamping the network in a hyperpolarized state for much of the time, and effectively regulating backpropagation of dendritic action potentials by shunting dendritic current. Without dendritic backpropagation, incoming parallel fiber input no longer generated an active event in the distal dendritic region and it is likely that this would significantly change the integration of synaptic input in the apical dendritic tree.

A number of studies have dissociated the amnestic and sedative-hypnotic effects of etomidate, showing that they are mediated through different subunits of the GABA_A receptor (Reynolds et al. 2003; Belelli et al. 2005; Cheng et al. 2006). The sedative effects of etomidate require the presence of β_2 or β_3 subunits while amnestic effects are associated with potentiation of tonic currents via the α_5 receptor subunit, but this action also requires the presence of β_2 or β_3 subunits in order for etomidate to bind to the receptor (Jurd et al. 2003; Wafford et al. 2004). Since both tonic and phasic effects of etomidate are seen in LG efferent cells and MG inhibitory interneurons, it seems likely that GABA_A receptors in these cells contain both the α_5 subunit and the β_2 or β_3 subunits, resulting in both tonic hyperpolarization and increased synaptic inhibition observed under etomidate anesthesia.

In terms of integrative function and the mechanism of sensory attenuation in anesthetic action, the effects of etomidate would be to increase response threshold and to constrain the timing of responses to excitatory input by potentiation of the following short-latency inhibition. While this would apparently increase the temporal precision of spike timing, it would also reduce the probability that either sensory input or descending activity alone could produce a network output. The overall effect would be to increase the “temporal contrast”, transmitting only the stronger network responses caused by coincidence of the highly synchronized, reafferent sensory
input generated by the fish’s own electric organ discharge with the precisely-timed descending
corollary discharge-driven excitatory input.
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Legends to the figures

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outwards through the molecular layer (traces a and b). Abbreviations: layers of the electrosensory lobe - df, deep fiber layer; int, intermediate layer; d gran, deep granular cell layer; s gran, superficial granular cell layer; plex, plexiform layer; ggl, ganglionic cell layer; mol, molecular layer.

**Fig. 3** Effects of etomidate and bicuculline on current sources (blue) and sinks (red) following stimulation in the deep fiber layer. Field potentials are superimposed on the current source density plots. Stimulus strength was four times threshold. An MG cell is depicted to the left, to relate neuron morphology to current flow through the structure. All figures are from the same slice, and the stimulus intensity was kept constant. This figure shows data that is representative of results obtained in 4 separate experiments. A: Responses to deep layer stimulation (SD; ▼) generate a sink in the deep fiber, intermediate and deep granular layers, that is associated with n pre and n post waves in the overlying field potentials. A double current source develops in the superficial granular, plexiform and ganglionic layers. A backpropagating source/sink/source constellation appears several milliseconds later in the molecular layer; this sink is associated with nl. Backpropagating activity is markedly depressed when a second, identical, stimulus is given 50 ms following the first one. The dotted white line in the molecular layer indicates the recording site of the field potentials shown in F. B: Etomidate strongly reduces backpropagating activity, in parallel to loss of the associated second part of the double current source in the granular layer. Etomidate has no effect on the sink in the deep layers. C. Bicuculline antagonizes the effects of etomidate. Backpropagation is restored and the sink in the deep layers is prolonged (white arrow), although etomidate alone had not reduced it previously. D. Washout of bicuculline. E. Washout of etomidate shows that the effects of etomidate are reversible. F.
Comparison of field potentials recorded in the molecular layer show strong reduction of n1 in the presence of etomidate, and that this effect is blocked by the addition of bicuculline.

**Fig. 4** Increasing the stimulus strength in the deep fiber layer from just above threshold to two times threshold restores the backpropagating sink that was previously inhibited by etomidate. A: Predrug control. B: Etomidate reduces backpropagating activity. The stimulus strength was identical to the one used in A. C: Doubling the stimulus strength restores backpropagating activity. All figures are from the same slice, and are representative of three separate experiments.

**Fig. 5** Field potentials recorded in the ELL, evoked by stimulation in the molecular layer. Field potentials are aligned with an MG cell to relate the field potentials to the cellular compartments of efferent neurons and interneurons with cell bodies in the ganglionic cell layer. Stimulation evokes three negative waves “on beam” with the parallel fiber input (trace a). n1 corresponds to the parallel fiber volley, n2 reflects postsynaptic activation in the apical dendrites, and n3 reflects backpropagating broad spikes generated in the ganglionic layer (trace b). A positive wave is observed in the plexiform and granular cell layer (trace c). A second identical stimulus given at 50 ms shows potentiation of ganglionic layer responses and of the backpropagating n3 (*). Abbreviations as in Fig. 2.

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**Fig. 7** Increasing the stimulus strength from just above threshold to two times threshold in the molecular layer restores the backpropagating sink that was previously reduced by etomidate. A: Predrug control. B: Etomidate reduces backpropagating activity. The stimulus strength was identical to that in A. C: Doubling the stimulus strength increased backpropagating activity, indicating that etomidate increases the firing threshold of backpropagating spikes that underlie the backpropagating sink. The white arrows in A and C denote the part of the backpropagating sink that links the sinks in the inner and outer molecular layer. In C this may be partly masked by the source following the stronger distal synaptic response (see explanation in Results). All figures are from the same slice, and these results are representative of three independent experiments.
**Fig. 8** The NMDA-receptor blocker AP-5 strongly reduces the amplitude of the late phase of the current sink in the outer molecular layer. A: Predrug control. B: In the presence of AP-5 the late phase of the sink in the outer molecular layer (white arrow) is strongly reduced, demonstrating that this part of the sink is NMDA-receptor dependent. Although the early (AMPA) component of excitatory response is unchanged, block of the NMDA-receptor mediated input reduces both the later component of the postsynaptic response and activation of the following disynaptic inhibitory input. Thus, although a backpropagating sink is visible in both A and B, the sink in the molecular layer appears slightly stronger in B. However, “on beam” amplification is much reduced. A and B show data from the same slice, and these results are representative of two independent experiments.

**Fig. 9** Effects of etomidate on membrane polarization and synaptic responses in MG, LF, and LG cells. A: Under etomidate, MG cells were tonically hyperpolarized (n = 9) and synaptic IPSPs evoked by molecular layer stimulation (▼) were strongly potentiated (control: black trace; etomidate: gray trace - black arrow). B: In an LF cell etomidate revealed an IPSP in response to parallel fiber stimulation (gray trace - black arrow) that was not obvious in the control situation (black trace), although no tonic hyperpolarization was observed. C: In LG cells with an EPSP – IPSP sequence to molecular layer stimulation (black trace), etomidate produced tonic hyperpolarization and potentiated parallel fiber IPSPs (gray trace – black arrow) (n = 8). D: Bicuculline blocked parallel fiber IPSPs in LG cells, and increased the probability of action potential firing. The cell illustrated, which is different from that shown in C, fired two or three action potentials following molecular layer stimulation under bicuculline (black trace, control; gray trace, bicuculline).
Fig. 10 The probability of action potential firing is reduced under etomidate by tonic hyperpolarization, by potentiation of IPSPs and by increased shunting inhibition. A1: In a control without etomidate, parallel fiber stimulation (▼) evoked an EPSP, a small spikelet and a backpropagating action potential followed by a disynaptic IPSP (arrow); intracellular stimulation evoked a small spikelet and a backpropagating broad spike. A2 and A3: Etomidate caused tonic hyperpolarization (4mV) and potentiated the disynaptic IPSP (arrow) to an amplitude of 7.9mV, shunting the synaptically-evoked backpropagating action potential and blocking the spiking response to intracellular depolarization. Scale bars in A3 also apply to A1 and A2.

B1 and B2. In the same cell under etomidate, increasing parallel fiber stimulation from 10µA (B1) to 15µA (B2; double ▼) and intracellular stimulation strength from 0.3 to 0.4nA (B1) and then to 0.8 nA (B2) restored spikelet and action potential generation. However, note that action potential amplitude is less than in the control (A1) due to shunting by the potentiated disynaptic IPSP (arrow). B3: In the absence of a preceding synaptically-evoked IPSP, an intracellular stimulus was similar to that in the predrug control (A1) evoked an action potential even at -67 mV, showing that tonic inhibition alone was not sufficient to block action potential generation.

B4: Balancing out tonic inhibition by depolarizing the membrane potential to the control value of -63 mV restored the spiking response to the synaptic stimulation, but not to intracellular stimulation demonstrating the effect of the potentiated IPSP (arrow).

C1-3. In an LG cell, an intracellular stimulus following parallel fiber stimulation at 20ms evoked four action potentials in control conditions (C1). Etomidate caused tonic hyperpolarisation (2mV), and potentiated the parallel fiber disynaptic IPSP (C2, arrow), reducing the response to the intracellular stimulus to a single spike. Increasing the delay of the intracellular pulse to 55 ms restored the initial multiple-spiking response (C3). The scale bars in C3 also apply to C1 and C2.
Fig. 11 Plastic responses of MG cells in the presence of etomidate or of etomidate together with bicuculline. Spike timing dependent plasticity was induced by repetitive association of parallel fiber stimulation (▼) with a postsynaptic intracellular current pulse following at a delay of 25 ms, sufficient to evoke backpropagating spikes. A: Control for the protocol to induce plasticity, in the presence of etomidate together with bicuculline to block synaptic inhibition. The parallel fiber EPSP was depressed after pairing of synaptic input with intracellularly evoked backpropagating spikes (n = 2). Responses are averages of 6 traces, immediately before pairing (black trace) and immediately after 5 min of pairing (gray trace). B: Under etomidate alone, parallel fiber stimulation evoked an EPSP followed by an IPSP. The parallel fiber EPSP amplitude was reduced following pairing and the following IPSP appeared earlier (n = 5 cells from 5 fish). (black trace, average of 6 traces immediately prior to pairing; gray trace, average of 6 traces immediately after the end of the pairing period). This cell is different from the one presented in A. C: Quantification of EPSP size, measured as the area under the EPSP trace showing the time course of plastic change. The data are from the same MG cell as that shown in B. Note that EPSP size gradually returns towards control values over 10 mins (see text and Han et al. 2000b). Black dots, control EPSP before pairing; open circles, EPSP during pairing; gray circles, EPSP following pairing.
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